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(71) Applicant *(for all designated States except US):* IMPERIAL CANCER RESEARCH TECHNOLOGY LTD [GB/GB]; Sardinia House, Sardinia Street, London WC2A 3NL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : TAYLOR-PAPADIMITRIOU, Joyce [GB/GB]; 9 Cedar Road, Berkhamsted, Herts HP4 2LA (GB). GENDLER, Sandra [US/GB]; 20 St James Mansions, West End Lane, West Hampstead, London NW6 2AA (GB). BURCHELL, Joy [GB/GB]; 4 Whites Cottages, Fletching, Uckfield, West Sussex TN22 3SP (GB).

(74) Agents: CRESSWELL, Thomas, Anthony, et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

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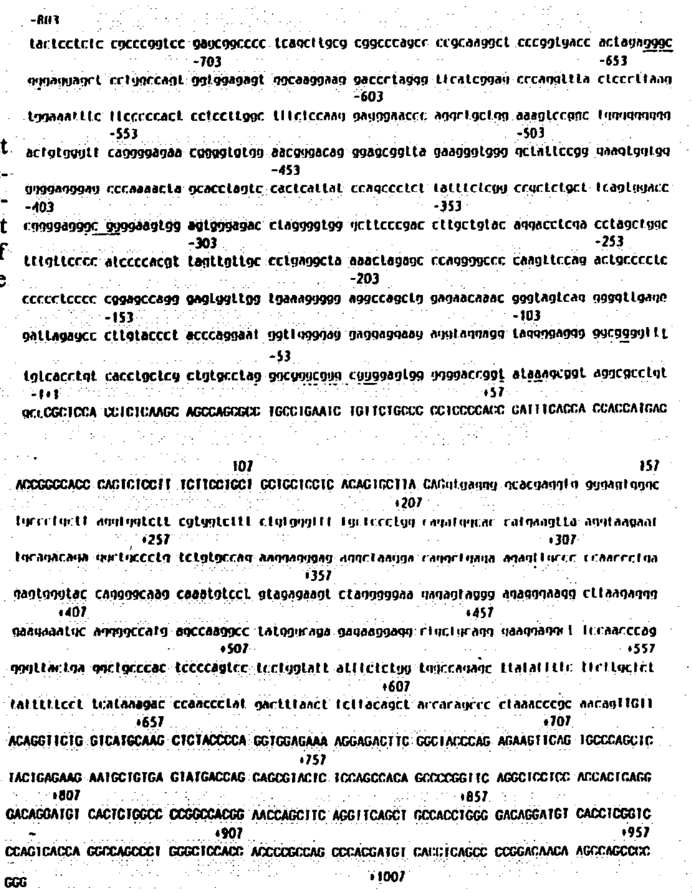
(57) Abstract

A nucleic acid fragment comprising a portion of at least 17 contiguous nucleotide bases which portion has a sequence the same as, or homologous to a portion of corresponding length of the sequence of the coding strand as set out in Fig. 1 or the same as, or homologous to a portion of corresponding length of the sequence complementary to the sequence of the coding strand set out in Fig. 1.



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MUCIN NUCLEOTIDES

The present invention relates to nucleotide fragments, polypeptides and antibodies and their use in medical treatment and diagnosis.

In International Patent Application no. WO-A-88/05054 there is disclosed a tandem repeat sequence contained in the human polymorphic epithelial mucin (HPEM) gene and nucleotide probes, polypeptides, antibodies and antibody-producing cells which are useful in the diagnosis and treatment of adenocarcinomas such as breast cancer.

The present inventors have now elucidated the nucleotide base sequence of the gene in the region 5' of the tandem repeat sequence (unless the context implies otherwise, directions such as "5'" or "3'", "upstream" or "downstream" used herein refer to the non-template strand of the genomic DNA or fragments thereof). The complete sequence of the 1763 nucleotide bases of the non-template strand upstream of and including the first SmaI restriction site in the tandem repeat is set out in Fig. 1. The sequence of 1575 nucleotide bases of the non-template strand upstream of and including the first SmaI restriction site in the tandem repeat as set out in Fig. 3 has been extended and some parts have been corrected in the light of repeat experiments. The template strand has a complementary sequence and it is this strand which is transcribed into RNA during expression of the gene

product.

In addition to conventional transcriptional and translational start sites and intron splicing sites, this sequence contains a number of features which may be important in the diagnosis and therapy of cancers and in expression of proteins from recombinant vectors. These features will be described below. The amino acid sequence corresponding to the translated portions of this nucleotide sequence gives rise to peptides and thence to antibodies and antibody-producing cells which may also be useful in such diagnosis and treatment.

In one aspect the present invention provides a nucleic acid fragment comprising a portion of at least 17 contiguous nucleotide bases which portion has a sequence the same as, or homologous to a portion of corresponding length of the sequence of the coding strand as set out in Fig. 1 or the same as, or homologous to a portion of corresponding length of the sequence complementary to the sequence of the coding strand set out in Fig. 1.

As used herein the term "fragment" is intended to include restriction endonuclease-generated nucleic acid molecules and synthetic oligonucleotides.

The nucleic acid fragments of the invention may be single-stranded or double-stranded and they may be RNA or DNA fragments. Single stranded fragments may be "plus" or coding strands having the sequence of Fig. 1 or a part

thereof or a sequence homologous thereto. Alternatively the single stranded fragments may be "minus" or non-coding strands having a sequence complementary to the sequence of Fig. 1 or a part thereof or a sequence homologous thereto. Double stranded fragments contain a complementary pair of strands, (ie. one plus strand and one minus strand).

RNA fragments according to the invention will, of course, contain uridylic acid ("U") residues in place of the deoxythymidylic acid residues ("T") of the coding (non-template) strand set out in Fig. 1 or, if complementary to the sequence of the coding strand, they will contain U residues in positions complementary to the adenylic acid ("A") residues in the coding strand set out in Fig. 1.

Preferably the nucleic acid fragments of the invention are double-stranded DNA fragments. Single-stranded nucleic acid fragments of the invention are at least 17 nucleotide bases in length. Double-stranded nucleic acid fragments of the invention are at least 17 nucleotide base pairs in length. Preferably the fragments are at least 20 bases or base pairs in length, more preferably at least 25 bases or base pairs and yet more preferably at least 50 bases or base pairs in length.

Statistically it is almost certain that a 17 nucleotide base sequence will be unique so that any

nucleic acid fragment having a contiguous portion of 17 nucleotides of a sequence identical to a portion of corresponding length of the coding strand as set out in Fig. 1, or the same as the non-coding strand complementary to the sequence of Fig. 1, will be new. Fragments according to the invention which are only 17 nucleotides or nucleotide bases in length have a sequence the same as, or complementary to, that set out in Fig.1. Longer fragments of the invention may have a sequence which is homologous to a corresponding portion of the sequence for the coding strand as set out in Fig. 1 or to the complementary non-coding strand.

Preferably nucleic acid fragments according to the invention have at least 75% sequence homology with a corresponding portion of the sequence of Fig. 1 or the complementary non-coding strand, for instance 80 or 85%, more preferably 90 or even 95% homology. Differences may arise through deletions, insertions or substitutions. In addition to containing a portion homologous to or the same as the sequence of the coding strand in Fig. 1 or complementary non-coding strand, the nucleic acid fragments of the invention may include sequences completely unrelated to that in Fig. 1.

Particular features of interest within the coding strand in Fig. 1 are set out in Tables 1 to 3 below:

TABLE 1: Signal Sequences

Location*	Sequence in PEM	Significance
1-2	CG	transcriptional start site
73-75	ATG	translational start signal
131-132	GT	start of first intron
631-632	AG	end of first intron
100-130 } and } 633-637 }	TTCCTGCTGCTGCT-CCTCACAGTGCTTA-CAG...TTGTT	Signal sequence, interrupted by first intron (first intron indicated by"...").
955-960	CCCGGG	SmaI site at start of tandem repeat

<u>Footnotes to Tables 1 and 2</u>

+ In the consensus sequences : R is A or G

N is A, C, G or T

W is A or T

X is

Y is C or T

* Locations are of the 5' base of the indicated PEM sequence numbered as in Fig. 1.

TABLE 2 : Regulatory elements within the 5' flanking sequence

Regulatory element	Consensus Sequence+	Sequence in PEM	Location*
SP1	GGGCGG	GGGCGG	-727
		GGGCGG	-397
		GGGCGG	-94
		GGGCGGGCGGGCGGG	-54
SV40 enhancer element			
a	ATGTGTGT	CTGTGGGT	-562
b	GCATGCAT	GCCTGCCT	+25
c	GTGGATAG	GTGGAGAG	-702
AP-1	CTGACTCA G A	GTGACCAC	-739
		CTGCTTCA	-418
		GTGCCTAG	-61
		CTGCCTGA	+27
AP-2	CCCCAGGC G G	ACCCAGGC	-597
		CACCGGGC	+77
NF1/CTF	TTGGCTNNNAGCCAA	TTGGCTTTCTCCAA	-618
Glucocorticoid regulatory element:			
Core sequence	TGTTCT	TGTTCT	+38
		TGTTCC	-321
Consensus sequence	GGTACANNNTGTTCT	GCCTGAATCTGTTCT	+29
		AGCTGGCTTTGTTCC	-330
CACCC factor	CACCC	CACCC	+54
		CACCC	+84
Progesterone receptor consensus sequence	ATTCCTCTGT	ACTCCTCTCC	-802
		ACTCCTCCTT	-626
		ATTTCTCGGC	-432
Estrogen consensus sequence	GGTCANNNTGACC	GCTCCCGGTGACC	-746
RNA Polymerase III			
Box A	RRYNNARYXGG	GACCTAGCTGG	-335
		AGTGGAGTGGG	-388
Box B	GWTCRANNC	GTTCCAGAC	-260
Enhancer sequences:			
Interferon-ß seq	GGAAATTCCTCTG	GGAAATTTCTTCC	-642
CMV enhancer	GGAAAGTCCCGTT	GGAAAGTCCGGCT	-585

The sequence in Fig. 1 also includes two sites occurring in the promoter region and in the first intron having 70 to 80% homology with the mammary consensus, sequence (Rosen, J.M. in "The Mammary Gland, Development, Regulation and Function", Ed. Nevill, M.C. and Daniel, C.W. Plenum Press, pp 301-322). These sites are set out in Table 3 below:

TABLE 3

Location	Sequence
	*** * *
-289 to -274	AGGCTAAAACTAGAGC
	* ** **
+230 to +245	GTAAGAATTGCAGACA
Consensus	RGAAGRAAANTGGACA

Positions are numbered in accordance with Fig. 1.

* indicates a mismatch with the consensus sequence.

In the consensus sequence:- R is A or G.

N is A, C, G or T.

Preferred fragments according to the present

invention include the transcriptional and translational start signals, "TATAA" box and at least one of the regulatory elements (transcription factor binding sites) set out in Table 2 above. More preferably these fragments contain 2 or more, for instance 3, 4 or 5 of the regulatory elements in addition to the TATAA box or even all of the regulatory elements set out in Table 2. Those fragments containing more than one of the regulatory elements of Table 2 preferably also preserve the relative spacings of those sites from one another and from the TATAA box and transcriptional and translational start signals.

Other preferred fragments of the invention contain at least one of the regions homologous to the mammary consensus sequences as set out in Table 3. Preferably these fragments contain both of the regions having homology with the mammary consensus sequences as set out in Table 3. Those fragments containing both regions having homology with the mammary consensus sequence preferably also preserve the relative spacing of those regions, as found in Fig. 1, from one another and from the TATAA box and transcriptional and translational start signals.

Yet further preferred fragments according to the invention comprise the TATAA box, the transcriptional and translational start signals, at least one and preferably two or more of the regulatory elements as set out in Table 2 and at least one and preferably both of the regions having

homology with the mammary consensus sequence as set out in Table 3. Yet more preferably these fragments also preserve the relative spacing of the features from Tables 1, 2 and 3. Particularly preferred fragments according to the invention comprise the sequence upstream of the TATAA box as set out in Fig. 1 together with, and downstream thereof, transcriptional and translational start signals and a polypeptide coding sequence in correct reading frame register with the promoter sequences and the TATAA box, transcriptional and translational start signals. The coding sequence may encode a part or parts of the polypeptide encoded by the mucin gene, for instance a part or parts thereof other than the tandem repeat sequence, or polypeptides unrelated to that encoded by the mucin gene.

Other particularly preferred fragments according to the present invention comprise promoter sequences, a TATAA box, transcriptional and translational start signals and, downstream thereof and in correct reading frame register therewith a coding sequence corresponding to a portion of the mucin gene, for instance corresponding to the first exon (corresponding to bases (1 to 130 of Fig.1.) or a part thereof and/or the second exon (corresponding to bases 633 onwards in Fig.1.) or a part thereof, for instance a part thereof other than the tandem repeat sequence as set out in WO-A-88/05054.

In an especially preferred aspect the fragments

contain (i) the first 26 bases (bases 1 to 26 of Fig. 1) or (ii) the whole of the first exon (bases 1 to 130 of Fig.1.) and/or (iii) the splicing/ligating sites for the first intron set out in Table 1 and a non-coding sequence between these sites. The non-coding sequence may be the same as or different to the sequence of the first intron as shown in Fig. 1. Preferably it is the same.

Other preferred fragments of the invention comprise at least a portion of the first intron (bases 231 to 632 of Fig. 1). Further preferred fragments of the invention comprise at least a portion of the 5'-flanking sequence upstream of base -423 of Fig. 1.

Other preferred fragments of the invention comprise a portion of the sequence of Fig. 1 corresponding to a portion of the sequence of Fig. 3.

Further preferred fragments of the invention comprise a combination of any two or more of the foregoing preferred features.

Fragments according to the present invention containing functional coding sequences for a least a part of the first or second exons set out in Fig. 1 are useful in the production of polypeptides corresponding to a part or all of the mucin gene product. Such polypeptides are, in turn useful as immunogenic agents for instance in active immunisation against Human Polymorphic Epithethial Mucin (HPEM) for the prophylactic or therapeutic treatment of

cancers or raising antibodies for use in passive immunisation and diagnosis of cancers. For use in such methods the fragment, which codes for a polypeptide chain substantially identical to a portion of the mucin core protein, may be extended at either or both the 5' and 3' ends with further coding or non-coding nucleic acid sequence including regulatory and promoter sequences, marker sequences, and splicing or ligating sites. Coding sequences may code for other portions of the mucin core protein chain (for instance, other than the tandem repeat) or for other polypeptide chains. The fragment according to the invention, together with any necessary or desirable flanking sequences is inserted, in an appropriate open reading frame register, into a suitable vector such as a plasmid, or cosmid or a viral genome (for instance vaccinia virus genome) and is then expressed as a polypeptide product by conventional techniques. In one aspect the polypeptide product may be produced by culturing appropriate cells transformed with a vector, harvested and used as an immunogen to induce active immunity against the mucin core protein [Tartaglia et al., Tibtech, 6, 43: (1988)].

Fragments according to the present invention incorporating regulatory elements of Table 2 and/or mammary consensus sequences of Table 3 may be used in securing tissue-specific expression of functional coding sequences in appropriate reading frame register downstream of the

regulatory elements and/or associataed with the mammary consensus sequences. Such fragments may therefore be used to express parts or the whole of the mucin gene or any other coding sequence in cells of epithelial origin. Applications of this are in therapy and immunisation where such fragments and associated coding sequences are administered to patients such that the coding sequence will be expressed in epithelial tissues leading to a therapeutic effect or an immune reaction by the patient against the polypeptides.

The fragments may be presented as inserts in a vector such as viral genomic nucleic acid and introduced into the patients by inoculation of the vector for instance as a modified virus. The vector then directs expression of the polypeptide _in vivo_ and this in turn serves as a therapeutic agent or as an immunogen to induce active immunity against the polypeptide. This strategy may be adopted, for instance, to secure expression of polypeptides encoded by the HPEM gene for treatment or prophylaxis of adenocarcinomas such as breast cancer or to secure tissue specific expression of other peptides under control of the regulatory sequences of Table 1, for instance by administration of a modified vaccinia virus containing the fragment and coding sequences in its genomic DNA. RNA fragments of the invention may similarly be used by administration via a retroviral vector. Selection of tissue specific virus vectors to carry the fragments of the

invention and coding sequences will further restrict expression of the polypeptide to desired target tissues.

Fragments of the invention may also be used to control expression of oncogenic proteins in experimental transgenic animals. Thus, for instance, a transgenic mouse having an oncogene such as ras, erbB-2 or int 2 expressed under control of the present tissue specific fragments may develop breast tumours and be useful in testing diagnostic agents such as tumour localisation and imaging agents and in testing therapeutic agents such as immunotoxins.
Nucleic acid fragments according to the invention are also useful as hybridisation probes for detecting the presence of DNA or RNA of corresponding sequence in a sample. For use as probes fragments are preferably labelled with a detectable label such as a radionuclide, enzyme label, fluorescent label or other conventional directly or indirectly detectable labels. For some applications, the probes may be bound to a solid support. Labelling of the probes may be achieved by conventional methods such as set out in Matthews et al., Anal. Biochem. 169: 1-25 (1988).

In further aspects, the present invention provides cloning vectors and expression vectors containing fragments according to the present invention. The vectors may be, for instance, plasmids, cosmids or viral genomic DNA. The present invention further provides host cells containing

such cloning and expression vectors, for instance epithelial cells transformed with functional expression vectors containing expressible fragments according to the invention.

The invention further provides nucleic acid fragments which encode polypeptides as defined below. Such fragments may be fragments as hereinbefore defined. However, in view of the redundancy of the genetic code, nucleic acid sequences which differ slightly or substantially from the sequence of Fig. 2 may nevertheless encode the same polypeptide.

The nucleic acid fragments of the invention may be produced de novo by conventional nucleic acid synthesis techniques or obtained from human epithelial cells by conventional methods, Huynh et al., "DNA Cloning: A Practical Approach" Glover, D.M. (Ed) IRL, Oxford, Vol 1, pp49-78 (1985).

The invention therefore also provides probes, vectors and transformed cells comprising nucleic acid fragments as hereinbefore defined for use in methods of treatment of the human or animal body by surgery or therapy and in diagnostic methods practiced on the human or animal body and for use in the preparation of medicaments for use in such methods. The invention also provides methods for treatment of the human or animal body by surgery or therapy and diagnostic methods practiced in vivo as well as ex vivo and in vitro which comprise administering such fragments, probes, vectors or

transformed cells in effective non-toxic amount to a human or other mammal in need thereof.

Processes for producing fragments according to the invention and probes, vectors and transformed cells containing them and processes for expressing polypeptides encoded by, or under the regulatory control of, fragments of the invention also form aspects of the invention.

The invention further provides a polypeptide comprising a sequence of at least 5 amino acid residues encoded by the coding portion of the DNA sequence as indicated in Fig. 2. Polypeptides according to the invention preferably have a sequence of at least 10 residues, for instance at least 15, more preferably 20 or more residues and most preferably all the residues shown in Fig. 2.

The polypeptide may additionally comprise N-terminal and/or C-terminal sequences not encoded by the DNA sequence indicated by Fig. 2.

Polypeptides of the invention containing more than 5 amino acid residues encoded by the DNA sequence in Fig. 2 may include minor variations by way of substitution, deletion or insertion of individual amino acid residues. Preferably such polypeptides differ at not more than 20% preferably not more than 10% and most preferably not more than 5% of residues in a contiguous portion corresponding to a portion of the sequence in Fig. 2.

The invention further provides polypeptides as

defined above modified by addition of a linkage sugar such as N-acetyl galactosamine on serine and/or threonine residues and polypeptides modified by addition of oligosaccharide moieties to N-acetyl galactosamine or via other linkage sugars. Optionally modified polypeptides linked to carrier proteins such as keyhole limpet haemocyanin, albumen or thyroglobulin are also within the invention.

Polypeptides according to the invention may be produced de novo by synthetic methods or by expression of the appropriate DNA fragments described above by recombinant DNA techniques and expressed without glycosylation in human or non-human cells. Alternatively they may be obtained by deglycosylating native human mucin glycoprotein (which itself may be produced by isolation from samples of human tissue or body fluids or by expression and full processing in a human cell line) [Burchell et al., Cancer Research, 47: 5467-5482, (1987), Gendler et al., P.N.A.S., 84: 6060-6064, (1987)], and digesting the core protein. The polypeptides of the invention are useful in active immunisation of humans, for raising antibodies in animals for use in passive immunisation, diagnostic tests, tumour localisation and, when used in conjunction with a cytotoxic agent, for tumour therapy.

The invention further provides antibodies against any of the polypeptides described above.

As used hereafter the term "antibody" is intended to include polyclonal and monoclonal antibodies and fragments of antibodies bearing antigen binding sites such as the $F(ab')_2$ fragments as well as such antibodies or fragments thereof which have been modified chemically or genetically in order to vary the amino acid residue sequence of one or more polypeptide chains, to change the species specific and/or isotype specific regions and/or to combine polypeptide chains from different sources. Especially in therapeutic applications it may be appropriate to modify the antibody by coupling the Fab, or complementarity-determining region thereof, to the Fc, or whole framework, region of antibodies derived from the species to be treated (e.g. such that the Fab region of mouse monoclonal antibodies may be administered with a human Fc region to reduce immune response by a human patient) or in order to vary the isotype of the antibody (see EP-A-0 239 400). Such antibodies may be obtained by conventional methods [Williams, Tibtech, 6:36, (1988)] and are useful in diagnostic and therapeutic applications, such as passive immunisation.

The term "antibodies" used herein is further intended to encompass antibody molecules or fragments thereof as defined above produced by recombinant DNA techniques as well as so-called "single domain antibodies" or "dAbs" such as are described by Ward, E.S. et al., Nature, 341:544-546

(1989) which are produced in recombinant microorganisms, such as Escherichia coli, harboring expressible DNA sequences derived from the DNA encoding the variable domain of an immunoglobulin heavy chain by random mutation introduced, for instance, during polymerise chain reaction amplification of the original DNA. Such dAbs may be produced by screening a library of such randomly mutated DNA sequences and selecting those which enable expression of polypeptides capable of specifically binding the polypeptides of the invention or HPEM core protein.

Antibodies according to the present invention react with HPEM core protein, especially as expressed by colon, lung, ovary and particularly breast carcinomas, but have reduced or no reaction with corresponding fully processed HPEM. In a particular aspect the antibodies react with HPEM core protein but not with fully processed HPEM glycoprotein as produced by the normal lactating human mammary gland.

Antibodies according to the present invention preferably have no significant reaction with the mucin glycoproteins produced by pregnant or lactating mammary epithelial tissues but react with the mucin proteins expressed by mammary epithelial adenocarcinoma cells. These antibodies show a much reduced reaction with benign breast tumours and are therefore useful in diagnosis and localisation of breast cancer as well as in therapeutic methods.

Further uses of the antibodies include diagnostic tests of assays for detecting and/or assessing the severity of breast, colon, ovary and lung cancers.

The antibodies may be used for other purposes including screening cell cultures for the polypeptide expression product of the human mammary epithelial mucin gene, or fragments thereof, particularly the nascent expression product. In this case the antibodies may conveniently be polyclonal or monoclonal antibodies.

The invention further provides antibodies linked to therapeutically or diagnostically effective ligands. For therapeutic use of the antibodies the ligands are lethal agents to be delivered to cancerous breast or other tissue in order to incapacitate or kill transformed cells. Lethal agents include toxins, radioisotopes and "direct killing agents" such as components of complement as well as cytotoxic or other drugs.

For diagnostic applications the antibodies may be linked to ligands such as solid supports and detectable labels such as enzyme labels, chromophores, fluorophores and radioisotopes and other directly or indirectly detectable labels. Preferably monoclonal antibodies are used in diagnosis.

Antibodies according to the present invention may be produced by inoculation of suitable animals with a polypeptide as hereinbefore described. Monoclonal

antibodies are produced by known methods, for instance by the method of Kohler & Milstein [Nature, 256: 495-497 (1975)] by immortalising spleen cells from an animal inoculated with the mucin core protein or a fragment thereof, usually by fusion with an immortal cell line (preferably a myeloma cell line), of the same or a different species as the inoculated animal, followed by the appropriate cloning and screening steps.

Antibody-producing cells obtained from animals inoculated with polypeptides of the invention and immortalised such cells form further aspects of the invention.

The invention further provides polypeptides, antibodies and antibody producing cells, such as hybridomas, as hereinbefore defined for use in methods of surgery, therapy or diagnosis practiced on the human or animal body or for use in the production of medicaments for use in such methods. The invention also provides a method of treatment or diagnosis which comprises administering an effective non-toxic amount of a polypeptide or antibody as hereinbefore described to a human or animal in need thereof.

Processes for producing polypeptides according to the invention whether by expression of nucleic acid fragments of the invention of otherwise, and for producing antibodies or fragments thereof and for producing antibody-producing cells such as immortalised cells, form further aspects of the

invention.

The invention further provides a diagnostic test or assay method comprising contacting a sample suspected to contain abnormal human mucin glycoproteins with an antibody as defined above. Such methods include tumour localisation involving administration to the patient of the antibody bearing detectable label or administration of an antibody and, separately, simultaneously or sequentially in either order, administering a labelling entity capable of selectively binding the antibody or fragment thereof. Diagnostic test kits are provided for use in diagnostic tests or assays and comprise antibody and, optionally, suitable labels and other reagents and, especially for use in competitive assays, standard sera.

The invention will now be illustrated with reference to the figures of the accompanying drawings in which:

Fig. 1. shows the deoxynucleotide base sequence of the 1763 bases upstream of and including the first SmaI restriction site in the tandem repeat sequence of WO-A-88/05054 using the conventional symbols A, C, G and T for the bases of the non-template strand. The base sequence is arranged in blocks of ten. Untranscribed sequence is in lower case, transcribed sequence is in upper case. The SP1 regulatory elements (Table 2), TATAA box, transcriptional and translational start sites (Table 1) are underlined.

Fig. 2. shows the sequence of the non-template strand commencing from the transcriptional start site, (residue 1 in Fig. 1.) and excluding the sequence of the first intron (bases 131 to 632 of the sequence in Fig.1.). Fig.2 also shows the predicted sequence of the polypeptide using the conventional 1 letter symbols for the amino acid residues. Amino acid residues are numbered down the left-hand side and nucleotide bases down the right hand side. The signal sequence is underlined. The sequences end at the first SmaI site in the tandem repeat.

Fig. 3. shows the deoxy nucleotide base sequence of the 1575 bases upstream of and including the first SmaI restriction site in the tandem repeat sequence of WO-A-88/05054 using the conventional symbols A, C, G and T for the bases of the non-template strand. The base sequence is arranged in blocks of ten in non-coding regions. The exon sequences are shown in blocks of three and translated codons are underlined. The start positions of exons 1 and 2, intron 1 and the signal sequence for exon splicing are numbered and labelled. Other features mentioned in Tables 1 and 2 are boxed. The sequence finishes with the first SmaI site of the tandem repeat sequence.

The present invention does not extend to fragments,

polypeptides and antibodies or related materials such as vectors and cells, which are specifically disclosed in WO-A-88/05054 or WO-A-90/05142, nor to the CDNA fragment whose sequence is indicated in Abe, M. et al., in Biochemical and Biophysical Research Communications, 165(2): 644-649 (1989).

The invention will now be illustrated by the following Examples:

EXAMPLE 1

In an attempt to obtain clones with 5' unique sequences, two gt10 libraries were screened with a probe for the tandem repeat. All the clones obtained lacked any non-repetitive sequence at the 5' terminus. Thus, a different strategy was adopted. To obtain 5' sequence we synthesized the cDNA corresponding to the 5' end of breast cancer cell line transcript using anchored-polymerise chain reaction (A-PCR). The A-PCR procedure [Loh, E.Y. et al., Science, 243: 217-220, (1989)] was used to synthesize cDNA corresponding to the 5' end of the transcript. For the 5' end clones total RNA (5 μg) prepared by the guanidinium isothiocyanate method [Chirgwin, J.M. et al., Biochem., 18: 5294-5299 (1979)] was used for first strand synthesis using a breast cancer cell line (BT20) transcript with AMV-reverse transcriptase (Life Sciences) in a 40 μl reaction mixture

[Okayama, H. and Berg, P., Mol. Cell. Biol., 2: 161-170 (1982)] containing 1 μg of an oligonucleotide primer made to the tandem repeat (5'CCAAGCTTGGAGCCCGGGGCCGGCCTGGTGTCCGG3'). The total RNA was subjected to reverse transcription, and the products were precipitated with spermine. A poly(dG) tail was introduced with terminal deoxy-transferase (500 U/ml, Pharmacia). Amplification was performed with Thermus aquaticus polymerise (Perkin Elmer Cetus) in 100 μl of the standard buffer supplied. The primers included the tandem repeat primer and for the poly(dG) end, a mixture of the AN polyC primer (5'GCATGCGCGCGGCCGCGGAGGCCCCCCCCCCCCCC3') and the AN primer (5'GCATGCGCGCGGCCGCGGAGGCC3') at a ratio of 1:9. Following an initial denaturation at 94^{o}C for 5 min, the reaction was annealed at 55^{o}C for 2 min, extended at 72^{o}C for 2.5 min and denatured at 94^{o}C for 1.5 min. Amplification was performed for 30 cycles, and the product was precipitated with ethanol. The DNA was sequentially cut with HindIII and SacII, separated on a 1.2% agarose Gel and the band of approximately 550 bp was purified onto DEAE membrane (Schleicher and Schuell), ligated into pBS-SK^{+} and transformed into bacteria XL-1 (Stratagene). This plasmid will be referred to as pBS-5'PEM. All restriction enzymes used were obtained from New England Biolabs Inc., oligonucleotide primers and probes were synthesized on an Applied Biosystems 380B DNA synthesizer.

Four colonies were selected for sequencing, and the

sequences agreed with each other and with sequence obtained from genomic clones of the region. A Leader sequence of 72 bp preceded the first ATG which was in-frame with the reading frame of the tandem repeat as previously determined (Fig. 1), and the sequence preceding first ATG, CCACCATGA, agrees with the Kozak consensus sequence (Kozak, M., Nucl. Acids. Res., 12: 857-872 (1984).

The primer extension technique was use to map precisely the position of the capsite. A 21 bp oligonucleotide primer (5'AGACTGGGTGCCCGGTGTCAT3') corresponding to nucleotides 73 to 93 ending at the A of ATG (Fig. 1) was end-labelled with [γ-^{32}P]ATP (> 5000 Ci/mmol, Amersham International plc) using T4 poly-nucleotide kinase (Pharmacia) and precipitated three times with equal volumes of 4 M ammonium acetate to remove free [γ-^{32}P]ATP from the kinased oligonucleotide. Labelled primer (1 x 10^5 dpm at 1 x 10^7 dpm/pmole) was annealed to 40 μg of total BT 20 RNA in 120 mM sodium chloride at 95^{o}C for 5 min, held at 65^{o}C for 1 h and cooled to room temperature. The annealed primer was extended using 18 units of reverse transcriptase in 50mM Tris pH 8.3 at 45^{o}C, 6 mM magnesium acetate, 10 mM dithiothreitol, 1.8 mM dNTPs in a total volume of 50 μl at 45^{o}C for 1h. The reaction was stopped by the addition of 50 mM EDTA and the RNA digested by treatment with RNase-A at 400μg/ml for 15 min at 37^{o}C. The samples were than phenol:chloroform extracted prior to ethanol precipitation

and electrophoresed on a standard 6% sequencing gel yielding two bands which mapped to two C's, 72 and 71 bases upstream of the ATG. The sequencing ladder was single-stranded control DNA (M13mp18) from the Sequenase kit (US Biochemical Corp.).

The most prominent product was 72 bp, equal to the number of base pairs from the 5' end of the oligonucleotide primer to the 5' end of the PCR-derived clone, thus confirming that the cDNA represents the entire length of its corresponding cellular mRNA 5' to the tandem repeat. The presence of a second band may be due to interference with reverse transcriptase by methylation of the C at base 71, since it forms a CpG dinucleotide. Under identical conditions, no primer extension product was seem using RNA from Daudi cells which do not express the PEM mucin.

<u>Cloning</u>

A plasmid library, grown in DH1αcells (RecA-), was used instead of a lambda library, because of the possibility of recombination occurring when lambda is grown in RecA+ cells. This recombination might have been expected, since a part of the tandem repeat sequence (GCTGGGGG) is closely related to the <u>chi</u> sequence (GCTGGTGG) of lambda phage which has been implicated as a hotspot for RecA-mediated recombination in <u>E.coli.</u>

Nucleotide sequence of cDNA clones

Fig 1. shows the DNA sequence from the 5' A-PCR-derived clone, including the consensus sequence of the tandem repeat. Sequences were determined in both directions. The region of conserved tandem repeats was not sequenced in full, although a cDNA tandem repeat clone obtained previously had been circularised, sonicated and about 40 clones sequences [(Gendler et al., J. Biol. Chem., 263:12820-12823 (1988)].

<u>Predicted amino acid sequence and composition of the PEM core protein</u>.

The core protein amino acid composition is dominated by the amino acid composition of the tandem repeat. Serine, threonine, proline, alanine and glycine account for about 60% of the amino acids.

The deduced sequence of the PEM core protein consists of distinct regions including (1) the N-terminal region containing a hydrophobic signal sequence and degenerate tandem repeats and (2) the tandem repeat region itself. At the N-terminus a putative signal peptide of 13 amino acids follows the first 7 amino acids. However, the actual site of cleavage has not been determined as attempts to obtain N-terminal sequence of the core protein were hindered by a blocked amino terminus. Following the signal sequence and preceding the first SmaI site (which is used to define the beginning of the tandem repeat region) are 107 amino acids. Greater than 50% of these amino acids comprise degenerate tandem repeats. Since the number of tandem repeats per molecule is large (greater than 21 for the smallest allele we have observed), this domain forms the major part of the core protein, and results in a highly repetitive structure which is extremely immunogenic [Gendler, S. <u>et</u> <u>al</u>., loc. cit]. The sequence of the 20

amino acid tandem repeat unit corresponds to what might be expected for a protein which is extensively O-glycosylated. Five serines and threonines, four of which are in doublets, are found in the repeat and these potential glycosylation sites are separated by regions rich in prolines (See Fig. 2).

CLAIMS

1. A nucleic acid fragment comprising a portion of at least 17 contiguous nucleotide bases which portion has a sequence the same as, or homologous to a portion of corresponding length of the sequence of the coding strand as set out in Fig. 1 or the same as, or homologous to a portion of corresponding length of the sequence complementary to the sequence of the coding strand set out in Fig. 1.

2. A fragment according to claim 1 comprising any one or more of the following:

(a) a signal sequence
TTCCTGCTGCTGCTCCTCACAGTGCTTACAGXTTGTT
wherein X is an optionally present intron

(b) a mammary consensus sequence AGGCTAAAACTAGACC

(c) a mammary consensus sequence GTAAGAATTGCAGACA

(d) a homologue of a sequence (a), (b) or (c) and

(e) a sequence complementary to a sequence (a), (b), (c) or (d).

3. A hybridisation probe comprising a fragment according to claim 1 or claim 2 bearing a detectable label or linked to a solid support.

4. A cloning or expression vector comprising a fragment

according to claim 1 or claim 2.

5. A transformed cell comprising a cloning or expression vector according to claim 4.

6. A polypeptide comprising a sequence of at least 5 contiguous acid residues encoded by the coding portion of the DNA sequence as indicated in Fig. 2.

7. An antibody against a polypeptide according to claim 6.

8. An antibody according to claim 7 bearing a detectable label or linked to a solid support.

9. An antibody-producing cell capable of secreting an antibody according to claim 7.

10. A diagnostic kit comprising a fragment according to claim 1 or claim 2 or a probe according to claim 3 or a polypeptide according to claim 6 or an antibody according to claim 7 or claim 8.

11. A fragment according to claim 1 or claim 2 or a probe according to claim 3 or a vector according to claim 4 or a cell according to claim 5 or claim 9 or a polypeptide

according to claim 6 or an antibody according to claim 7 or claim 8 for use in a method of treatment or diagnosis practised on the human or animal body.

12. Use of a fragment according to claim 1 or claim 2 or a probe according to claim 3 or a vector according to claim 4 or a cell according to claim 5 or claim 9 or a polypeptide according to claim 6 or an antibody according to claim 7 or claim 8 in the preparation of a medicament for use in a method of treatment or diagnosis practised on the human or animal body.

13. A method of treatment or diagnosis comprising administering to a cancer patient in need thereof or suspected to have a cancer an effective non-toxic amount of a fragment according to claim 1 or claim 2 or a probe according to claim 3 or a vector according to claim 4 or a cell according to claim 5 or claim 9 or a polypeptide according to claim 6 or an antibody according to claim 7 or claim 8.

14. A method of diagnosis comprising contacting a sample from a patient with a fragment according to claim 1 or claim 2 or a probe according to claim 3 or a vector according to claim 4 or a cell according to claim 5 or claim 9 or a polypeptide according to claim 6 or an antibody according to claim 7 or claim 8.

```
-803                                                   -753
tactcctctc cgcccggtcc gagcggcccc tcagcttgcg cggcccagcc ccgcaaggct cccggtgacc actagagggc
                      -703                                                   -653
gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcatcggag cccaggttta ctcccttaag
                                            -603
tggaaatttc ttcccccact cctccttggc tttctccaag gagggaaccc aggctgctgg aaagtccggc tggggggggg
           -553                                                   -503
actgtgggtt caggggagaa cggggtgtgg aacgggacag ggagcggtta gaagggtggg gctattccgg gaagtggtgg
                                 -453
ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcgg ccgctctgct tcagtggacc
-403                                                   -353
cggggagggc ggggaagtgg agtgggagac ctaggggtgg gcttcccgac cttgctgtac aggacctcga cctagctggc
                      -303                                                   -253
tttgttcccc atccccacgt tagttgttgc cctgaggcta aaactagagc ccaggggccc caagttccag actgcccctc
                                            -203
ccccctcccc cggagccagg gagtggttgg tgaaaggggg aggccagctg gagaacaaac gggtagtcag ggggttgage
           -153                                                   -103
gattagagcc cttgtaccct acccaggaat ggttggggag gaggaggaag aggtaggagg taggggaggg ggcggggttt
                                 -53
tgtcacctgt cacctgctcg ctgtgcctag ggcgggcggg cggggagtgg ggggaccggt ataaagcggt aggcgcctgt
 -1+1                                                        +57
gccCGCTCCA CCTCTCAAGC AGCCAGCGCC TGCCTGAATC TGTTCTGCCC CCTCCCCACC CATTTCACCA CCACCATGAC
```

Fig.1

```
                                  107                                                                      157
ACCGGGCACC CAGTCTCCTT TCTTCCTGCT GCTGCTCCTC ACAGTGCTTA CAGgtgaggg gcacgaggtg gggagtgggc
                                                           +207
tgccctgctt aggtggtctt cgtggtcttt ctgtgggttt tgctccctgg cagatggcac catgaagtta aggtaagaat
                 +257                                                                  +307
tgcagacaga ggctgccctg tctgtgccag aaggagggag aggctaagga caggctgaga agagttgccc ccaaccctga
                                            +357
gagtgggtac caggggcaag caaatgtcct gtagagaagt ctagggggaa gagagtaggg agagggaagg cttaagaggg
     +407                                                       +457
gaagaaatgc aggggccatg agccaaggcc tatgggcaga gagaaggagg ctgctgcagg gaaggaggct tccaacccag
                                 +507                                                        +557
gggttactga ggctgcccac tccccagtcc tcctggtatt atttctctgg tggccagagc ttatattttc ttcttgctct
                                                       +607
tatttttcct tcataaagac ccaaccctat gactttaact tcttacagct accacagccc ctaaacccgc aacagTTGTT
                 +657                                                                  +707
ACAGGTTCTG GTCATGCAAG CTCTACCCCA GGTGGAGAAA AGGAGACTTC GGCTACCCAG AGAAGTTCAG TGCCCAGCTC
                                            +757
TACTGAGAAG AATGCTGTGA GTATGACCAG CAGCGTACTC TCCAGCCACA GCCCCGGTTC AGGCTCCTCC ACCACTCAGG
     +807                                                       +857
GACAGGATGT CACTCTGGCC CCGGCCACGG AACCAGCTTC AGGTTCAGCT GCCACCTGGG GACAGGATGT CACCTCGGTC
                                 +907                                                        +957
CCAGTCACCA GGCCAGCCCT GGGCTCCACC ACCCCGCCAG CCCACGATGT CACCTCAGCC CCGGACAACA AGCCAGCCCC

GGG                                                    +1007
```

Fig.1 Cont'd

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```
                                                      CCG CTC CAC CTC TCA AGA GCC AGC GCC TGC CTG AAT   36

     CTG TTC TGC CCC CTC CCC ACC CAT TTC ACC ACC ACC ATG ACA CCG GGC ACC CAG TCT CCT   96
                                                          T   P   G   T   Q   S   P

                   SIGNAL SEQUENCE
     TTC TTC CTG CTG CTG CTC CTC ACA GTG CTT ACA GTT GTT ACA GGT TCT GGT CAT GCA AGC   156
 8    F   F   L   L   L   L   L   T   V   L   T   V   V   T   G   S   G   H   A   S

     TCT ACC CCA GGT GGA GAA AAG GAG ACT TCG GCT ACC CAG AGA AGT TCA GTG CCC AGC TCT   216
28    S   T   P   G   G   E   K   E   T   S   A   T   Q   R   S   S   V   P   S   S

     ACT GAG AAG AAT GCT GTG AGT ATG ACC AGC AGC GTA CTC RCC AGC CAC AGC CCC GGT TCA   276
48    T   E   K   N   A   V   S   M   T   S   S   V   L   S   S   H   S   P   G   S

     GGC TCC TCC ACC ACT CAG GGA CAG GAT GTC ACT CTG GCC CCG GCC ACG GAA CCA GCT TCA   336
68    G   S   S   T   T   Q   G   Q   D   V   T   L   A   P   A   T   E   P   A   S

     GGT TCA GCT GCC ACC TGG GGA CAG GAT GTC ACC TCG GTC CCA GTC ACC AGG CCA GCC CTG   396
88    G   S   A   A   T   W   G   Q   D   V   T   S   V   P   V   T   R   P   A   L

     GGC TCC ACC ACC CCG CCA GCC CAC GAT GTC ACC TCA GCC CCG GAC AAC AAG CCA GCC CCG   456
108   G . S   T   T   P   P   A   H   D   V   T   S   A   P   D   N   K   P   A   P
     SmaI
     GGC
      G
```

Fig. 2

```
-608                                                                    -538
TTGCTTCTCC  AAGAAGGGAA  CCCAGGTCGC  TGAAAGTCCG  GCTGGGCGGG  ACTGTGGGTT  TACGGGTAGA

-537                                                                    -468
ACTGCGTGTG  GAACGGAACG  GGAGCGGTTA  GAAGGGTGGG  GCTATTCCGG  AAGTGGTGGG  GGGAGGGAGC

-467                                                                    -398
CCAAAACTAG  CACCTAGTCC  ACTCATTATC  CAACCGTCTT  ATTTCTCGGC  CGCTCTGCTT  CAGTGGACCC

-388                                                                    -328
GGGGAGGGGC  GGGGAAGTGG  AGTGGGAGAC  CTAGGGGTGG  GCTTCCCGAC  CTTGCTGTAC  AGGACCTCGA

-327                                                                    -258
CCTAGCTGGC  TTTGTTCCCC  ATCCCCACGT  TAGTTGTTGC  CCTGAGGCTA  AAACTAGACC  GCAGGGGCCC

-257                                                                    -188
CAAGTTCCAG  ACTGCCCTCC  CCCTCCCCGG  AGCCAGGGAG  TGGTTGGTGA  AAGGGGAGGC  CAGCTGGAGA

-187                                                                    -118
ACAAACGGGT  AGTCAGGGGG  TTGAGCAGTT  AGAGCCCTTG  TACCCTACCC  AGGAATGGTT  GGGAGGAGGA

-117                                                                     -48
GGAAGAGGTA  GGAGGTAGGG  GAGGGGGCGG  GGTTTTGTCA  CCTGTCACCT  GCTCGCTGTG  CCTAGGGCGG
```

Fig.3

```
-47                                                        1      1
GCGGGCGGGG  AGTGGGGGGA  CCGGTATAAA  GCGGTAGGCG  CCTGTGC       CCG CTC CAC CTC TCA ACA GCC

                                                                 73
AGC GCC TGC CTG  AAT CTG TTC TGC CCC CTC  CCC ACC  CAT TTC ACC ACC ACC ATG ACA  CCG  GGC ACC CAG

      97        SIGNAL  SEQUENCE                                131  INTRON  1
TCT  CCT TTC TTC CTG  CTG  CTG CTC  CTC ACA GTG CTT  ACA G      GT GAGGGGCAC

GAGGTTGGGG  AGTTGGGCTT  GCCCTTGCTT  AGGTTGGTCT  TCGTTGGTTC  TTTCTGTGGG  CTTTTGCTCC

CTGGCAGATG  GCACCATGAA  GTTAAGGTAA  GAATATCAGA  CAGAGGCTGC  CCTGTCTGTG  CCAGAAGGAG

GGAGAGGCTA  AGGACAGGCT  GAGAAGAGTT  GCCCCCAACC  CTGAGAGTGG  GTACCAGGGG  CAAGCAAATG

TCCTGTAGAG  AAGTCTAGGG  GGAAGAGAGT  AGGGAGAGGG  AAGGCTTAAG  AGGGGAAGAA  ATGCAGGGGC

CATGAGCCAA  GGCCTATGGG  CAGAGAGAAG  GAGGCTGCTG  CAGGGAAGGA  GGCTTCCAAC  CCAGGGGTTA
```

Fig.3 Cont'd(1)

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CTGAGGCTGC CCACTCCCCA GTCCTCCTGG TATTATTTCT CTGGTGGCCA GAGCTTATAT TTTCTTCTTG

CTCTTATTTT TCCTTCATAA AGACCCAACC CTATGACTTT AACTTCTTAC AGCTACCACA GCCCCTAAAC

640 641 EXON 2

CCGCAAC AG TT GTT ACA GGT TCT GGT CAT GCA AGC TCT ACC CCA GGT GGA GAA AAG GAG

ACT TCG GCT ACC CAG AGA AGT TCA GTG CCC AGC TCT ACT GAG AAG AAT GCT GTG AGT ATG ACC AGC AGC

GTA CTC TCC AGC CAC AGC CCC GGT TCA GGC TCC CCA CCA CTC AGG GAC AGG ATG TCA CTC TGG CCC CGG

CCA CGG AAC CAG CTT CAG GTT CAG CTG CCA CCT GGG GAC AGG ATGTCA CCT CGG TCC CAG TCA CCA GGA

968

GCC CTG GGC TCC ACC ACC GCG CCA GCC CAC GAT GTC ACC TCA GCC CCG GAC AAC AAG CCA GCC CCG GG

----→ TANDEM REPEAT

Fig. 3 Cont'd (2)

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02020

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 H 21/04, C 07 K 13/00, C 12 N 15/12, C 12 P 21/00, C 12 Q 1/68, G 01 N 33/574, C 07 K 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N, C 12 P, C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 8805054 (IMPERIAL CANCER RESEARCH TECHNOLOGY) 14 July 1988 see the whole document; especially claims cited in the application	6
Y		3-5,7-10,14
	--	
P,X	Journal of Biological Chemistry, vol. 265, no. 10, 5 April 1990, The American Society for Biochemistry and Molecular Biology, Inc., (US), M.J.L. Ligtenberg et al.: "Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini", pages 5573-5578 see the whole article	1,2,6-9
P,Y		3-5,10,14
	--	
	./.	

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd April 1991	16 MAY 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Journal of Biological Chemistry, vol. 265, no. 25, 5 September 1990, The American Society for Biochemistry and Molecular Biology, Inc., (US), S.J. Gendler et al.: "Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin", pages 15286-15293 see figure 1 --	1,2,6
P,X	Journal of Biological Chemistry, vol. 265, no. 25, 5 September 1990, The American Society for Biochemistry and Molecular Biology, Inc., (US), M.S. Lan et al.: "Cloning and sequencing of a human pancreatic tumor mucin cDNA", pages 15294-15299 see the whole article --	1,2,6-9
P,A	WO, A, 9005142 (IMPERIAL CANCER RESEARCH TECHNOLOGY) 17 May 1990 see abstract and claims cited in the application -----------	1-10,14

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers XX because they relate to subject matter not required to be searched by this Authority, namely:

Claims 11-13

Pls. see Rule 39.1 (iv) - PCT:

Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers ______, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ______, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9002020
SA 43255

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/05/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8805054	14-07-88	AU-A- 1103988	27-07-88
		EP-A- 0341252	15-11-89
		JP-T- 2501828	21-06-90
WO-A- 9005142	17-05-90	None	

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82